Biocatalytic Self-Assembly Using Reversible and Irreversible Enzyme Immobilization

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Abstract

Biocatalytic control of molecular self-assembly provides an effective approach for developing smart biomaterials, allowing versatile enzyme-mediated tuning of material structure and properties as well as biomedical applications. We functionalized surfaces with bioinspired polydopamine and polyphenol coatings to study the effects of enzyme surface localization and surface release on the self-assembly process. We show how these coatings could be conveniently used to release enzymes for bulk gelation as well as to irreversibly immobilize enzymes for localizing the self-assembly to the surface. The results provide insights to the mode of action of biocatalytic self-assembly relevant to nanofabrication and enzyme-responsive materials.

Self-assembly of peptides and peptide derivatives is attracting interest as an effective bottom-up approach for the design of functional nanomaterials. [1-2] This interest has been motivated by the number of existing and potential applications that these materials may have, including catalysis, tissue engineering, controlled release and drug delivery. [3-4] Inspired by biological systems where self-assembly is often regulated by coupling to enzyme catalyzed formation or break-down of self-assembling building blocks, significant applications in this field exploit enzymes (or non-enzymatic catalysts) to trigger the formation of small-molecule building blocks that can self-assemble or reconfigure supramolecular aggregates. [5-8]

A number of enzymes have been utilized in this context, including phosphatases, esterases and proteases. [7, 9-10] These catalysts are typically dissolved and mixed with self-assembly precursors to enable structure formation over time. The action of surface immobilized enzymes has also been explored. Williams *et al*. first reported on the immobilization (using glutaraldehyde) of

thermolysin on an amine functionalized glass surface to enable self-assembly of Fmoc-protected peptides on a surface. [11] This was subsequently followed by the use of a polydopamine coating for enzyme immobilization, which resulted in the formation of a bulk gel in the container that the surface was immersed in. [12] More recently, Vigier-Carriere *et al*. employed alkaline phosphatase non-covalently immobilized in a polyelectrolyte multilayer to trigger the self-assembly and gelation of a Fmoc-protected tripeptide. [13]

Xu and co-workers exploited endogenously expressed enzymes that are either localized at cell surfaces or in specific subcellular spaces. Depending on the location of the enzymes overexpressed in disease-causing cells, self-assembly and gelation of peptide derivatives could be triggered in the intracellular or pericellular space, subsequently inducing cell apoptosis. [14-15] In a similar approach, Pires *et al*. employed cell surface bound alkaline phosphatase (ALP) to convert a simple carbohydrate derivative into a hydrogelator that assembles into a localized nanofibrous mesh around the cells. In this work, it was found that cell surface bound network formation and consequent apoptosis is induced only by membrane-bound, and not secreted, ALP [16] , suggesting that the location of the enzyme has a substantial impact on the assembly process. In a recent paper, Xu *at al*. also exploited localized endogenous ALP activity on a peptide derivative that self-assembles upon de-phosphorylation and generates fluorescence, allowing profiling of ALP activity in live cells. [17] Locally secreted endogenous enzymes (matrix metalloproteases, MMPs) can trigger the reconfiguration of peptide micelles loaded with doxorubicin to localize formation of fibrous structure and confine the release of the drug payload at the site of enzyme overexpression, providing a means to achieving site-specific anti-tumoral therapies. [18]

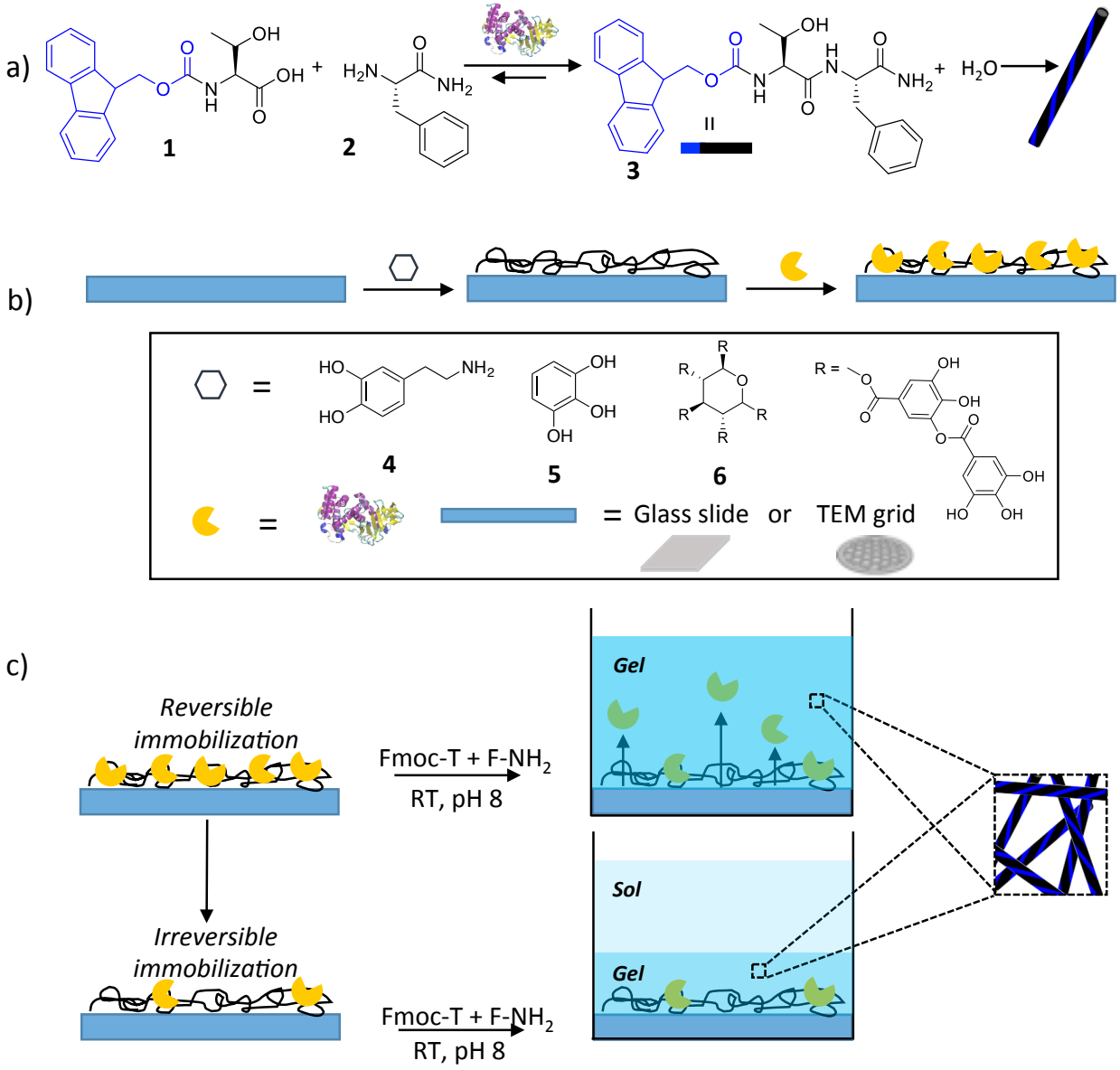

Figure 1 (a) Conversion of the pre-gelators Fmoc-T (**1**) and F-NH_2 (**2**) into the gelator Fmoc-TF-NH_2 (**3**) catalyzed by thermolysin. (b) Surface modification with polydopamine or polyphenols and subsequent enzyme immobilization (In the box: structure of dopamine (**4**), pyrogallol (**5**) and tannic acid (**6**) and the structure of thermolysin). The number of galloyl moieties per tannic acid can range from 2 to 12 depending on the plant source. We show penta-m-digalloyl-glucose from oak gall nuts. (c) Reversible and irreversible enzyme immobilization on modified surfaces for bio-catalytic self-assembly. The enzymes reversibly bound to the surface catalyze the coupling of the pre-gelators Fmoc-T (**1**) and F-NH_2 (**2**) and the formation of a bulk gel. The enzymes irreversibly bound to the surface retain activity after the additional washing procedure and are able to catalyze the conversion of the pre-gelators only at the surface and enable subsequent localized self-assembly of the gelator Fmoc-TF-NH_2 into nanofibers.

Clearly, there are a number of examples of biocatalytic self-assembly where enzymes are variously exploited as freely dissolved, compartmentalized, or surface-attached species. In particular, the aforementioned examples in biologically interfacing systems and in nanofabrication highlight the potential of using localized bio-catalytic self-assembly to enable complex (biological) function, by generating specific structural configurations through spatially selective nucleation and structure growth. We are therefore motivated to further investigate the strategy of enzyme immobilization for achieving spatially selective self-assembly. In this manuscript, we report a simple procedure to control the "reversibility" of enzyme immobilization and hence control the level of spatial localization of the self-assembly process.

We identified polydopamine and the novel polyphenol coatings as constituting a mild and versatile aqueous approach to surface immobilization that may be applied across diverse materials with minimal modification to the processing protocols. [19-21] While polydopamine coatings have been widely used to immobilize various proteins, [20] we report herein the first demonstration of immobilizing enzymes using a polyphenol coating. Dopamine (Fig. 1) spontaneously polymerizes in aqueous solutions under mildly oxidizing conditions to form thin polymeric films that are able to adhere to many types of surfaces without surface preparation and on substrates with complex geometries. The catechol (dihydroxyphenyl) groups of the polydopamine film confer a latent activity towards nucleophiles, that can be exploited for the conjugation of amine-containing biomolecules. [22-24] Recently, with the Messersmith group, we investigated a similar approach using plant polyphenols for the deposition of thin surface coatings on a variety of substrates. [21] Polyphenols are a large family of compounds that include epicatechin gallate (ECG), epigallocatechin (EGC), and tannic acid (TA), and the range of

potential coating precursors holds promise for an expansive range of properties. In contrast to polydopamine, polyphenol coatings may be deposited at and around neutral pH and have good transparency at visible wavelengths. The constituent phenolic units, including catechol and trihydroxyphenyl (galloyl) groups, are proposed to participate in reactions similar to those contributing to polydopamine formation, and should allow for the conjugation of biomolecules on polyphenol coated surfaces. [25] For this study, three different building blocks have been investigated to compare the efficacy of using polydopamine and polyphenol coatings for enzyme immobilization: dopamine (**4**), pyrogallol (**5**) and tannic acid (**6**) (Fig. 1).

To demonstrate that the findings translate across different surface types, and to facilitate the characterization of nanofiber self-assembly, glass cover slips and TEM carbon-coated copper grids were chosen as the support materials. The polydopamine and polyphenol coatings were deposited by simple immersion of the solid supports into a precursor solution for 2h at room temperature (see ESI). The modified solid supports were subsequently dipped in a 1 mg/mL thermolysin solution overnight to allow for enzyme immobilization.

The solid supports with immobilized thermolysin were used to control the location of the self-assembly of peptide amphiphiles. The modified amino acids Fmoc-T (**1**) and F-NH_2 (**2**) were employed as non-assembling precursors, which couple to form the gelator Fmoc-TF-NH_2 (**3**) upon thermolysin action (Fig. 1a). Fmoc-TF-NH_2 forms in high yield and self-assembles into fibrous structures, which in turn entangle to form supramolecular hydrogels. [26-27]

After thermolysin immobilization, the solid supports were treated using two different rinsing procedures (a milder or a more thorough wash, see ESI section 2 and 4) and subsequently immersed in the pre-gelator solution.

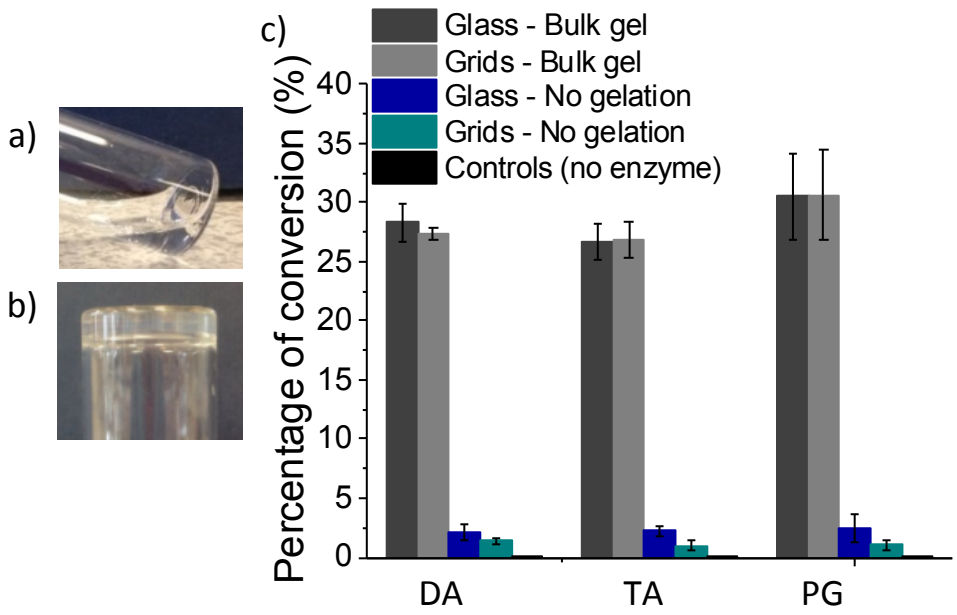

Figure 2 a) A glass cover slip with immobilized thermolysin is placed in a vial (immobilized enzyme side up) and dipped in a solution of pre-gelators. b) Formation of a hydrogel on top of the slide with the immobilized enzyme. c) HPLC analysis of the gels formed on top of both the modified glass cover slips and TEM grids after 4 days indicates the conversion of the pre-gelators **1** and **2** to **3**. Using a mild rinsing procedure, bulk gelation was observed on samples functionalized with all three immobilizing agents (**4**, **5** and **6**). When a more thorough wash was employed, bulk gelation was not observed and less than 3% conversion of the gel precursors was detected in the supernatant for all samples.

We observed macroscopically different results depending on the surface rinsing. When the surfaces were treated with the milder wash, all three immobilizing agents were effective in producing enzyme functionalized surfaces that catalyzed the condensation of the pre-gelators and subsequent gelation in the bulk phase, as demonstrated by standard tube inversion test and by reversed-phase HPLC analysis [28] (Fig. 2 and Fig S1). Similar results were obtained whether glass cover slips or TEM grids were used as the solid support (see Fig. S7). Bulk gelation was

observed within 3 to 4 days for all samples and HPLC analysis showed that the yield of pre-gelator conversion into the assembling species after gelation was similar in each case (~30%). In comparison, thermolysin dissolved in a bulk pre-gelator solution at a (typically high) concentration of 1 mg/mL catalyzes gelation faster, reaching ~80% conversion after 4 h. [26-27] It is likely that the longer gelation times and the lower conversion obtained from the immobilized enzyme are due to the lower overall amount of enzymes available (see enzyme assay below). The similar yields of conversion obtained when using glass cover slips or TEM grids suggest that the total amount of immobilized enzymes may be similar for the two supports.

In contrast to these results, when the surfaces with the immobilized enzymes were treated with further rinsing before reaction with the pre-gelators, no bulk gelation was observed (see FRET assay below). HPLC analysis of the supernatant confirmed that less than 3% of the amino acid precursors were converted within the bulk liquid phase (Figures 2 and S6). Similar results were obtained when the surface modification and enzyme immobilization were performed directly on TEM grids (Fig. S7).

We hypothesized that depending on the surface treatment upon enzyme immobilization we could achieve either a semi-reversible enzyme immobilization (which triggers the bulk gelation of the pre-gelators when enzymes desorb from the surface), or irreversible enzyme immobilization where no bulk gelation was observed. We were therefore interested in probing the enzyme release form the polymeric surface coatings and further investigating the surface effects in the case of irreversibly immobilized enzymes.

A Förster resonance energy transfer (FRET) fluorescence assay was employed to sensitively and directly probe the proteolytic activity of thermolysin during sequential washing steps. The custom-made peptide E(EDANS)-GT↓LGK(DABCYL) was employed as the enzyme substrate.

Fluorescence of the EDANS donor at the N-terminus is initially quenched via FRET by the DABCYL acceptor at the C-terminus. EDANS fluorescence is restored upon enzymatic cleavage at the T↓L position of the peptide to separate EDANS from the DABCYL acceptor. Proteolytic activity can hence be monitored by recording the fluorescence intensity *vs* time (measurement at the EDANS maximum emission wavelength: 510 nm).[29] Since the three immobilizing agents gave similar HPLC results in terms of yield of bio-catalytic self-assembly on both glass cover slips and TEM grid solid supports, only the results for polydopamine are shown below. (See ESI for additional results about the other surface coatings).

After enzyme immobilization, we initially applied the routine "milder" wash mentioned above, in which the solid supports were individually rinsed three times in ultrapure water. The samples were then immersed in 100 mM pH 8 phosphate buffer and placed on an orbital shaker for 30 min to aid the solubilization of any physically adsorbed enzyme. This procedure was repeated until no activity was observed in the wash solution.

Figure 3 shows that the first additional buffer immersion was able to release a significant amount of enzymes from the functionalized surface, while only a very small amount of enzymes was released in a second immersion. The solution from the first immersion produced a rapid increase in fluorescence before reaching a plateau after 20 min, indicating the presence of desorbed enzymes that were able to convert of all the substrate present within 15 min. Similar results were obtained for the surfaces modified with tannic acid and pyrogallol (Fig. S2). Quantitative analysis of the fluorescence kinetics shows a thermolysin concentration of 8.3×10^{-3} mg/ml (see Fig. S4). In contrast, the solution obtained after the second buffer immersion was able to produce only a negligible increase in the fluorescence signal that was detected only when the emission data was recorded overnight (Fig. 3b), corresponding to a thermolysin concentration

of 6.6 x 10^{-7} mg/ml, 4 orders of magnitude lower than in the first immersion/wash solution. The control (substrate solution without addition of any wash solution or solid sample) showed no activity overnight.

The FRET assay was also performed on the functionalized glass cover slip that had been subjected to the two stages of buffer immersion, by placing the glass sample directly in the fluorescence measurement chamber filled with a solution containing the substrate (see Sections 6 and 7.2 in ESI). In contrast to the wash solutions and the control, a steady increase in fluorescence was observed (Fig. 3a: "glass surface" data). This indicates that enzymes must be immobilized on the functionalized glass surface after the various washing steps, and the converted EDANS enzyme product could diffuse from the surface into the bulk solution to be detected.

Overall, the FRET experiment showed that there were two initial populations of enzymes on the functionalized surface after the "milder" routine rinse - reversibly and irreversibly attached - that could be differentiated simply using the additional buffer immersion procedure. These results are also in agreement with our hypothesis that the formation of a bulk hydrogel is due to the reversibly-bound enzymes released from the polyphenol/polydopamine coatings. It is assumed that the enzymes detected in the wash solutions were initially physisorbed on the surface. The majority of these reversibly attached enzymes could be desorbed during the first 30 min wash and in the second wash only a negligible amount of enzyme was released from the surface. After this 2-step washing/immersion procedure, a significant amount of irreversibly immobilized enzymes remained on the surface. The fluorescence kinetics show substantial surface activity equivalent to what would be observed for a dissolved enzyme concentration of 2.7 x 10^{-5} mg/ml. Given our spectrometer cuvette volume of 1 mL and a sample surface area of

0.66 cm^2, the measured activity indicates an active immobilized thermolysin surface concentration of 40 ng/cm^2. In comparison, a monolayer of thermolysin corresponds to 98 ng/cm^2 (see Fig. S4).

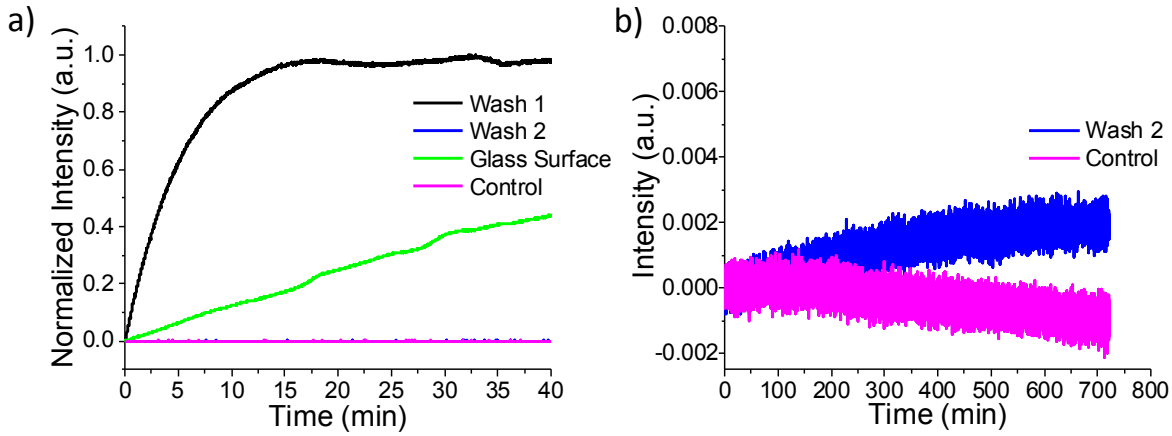

Figure 3 a) FRET assay to detect thermolysin activity in the wash solution and for the enzyme immobilized on the surface of a glass cover slip modified with polydopamine. In the first wash solution (black) the fluorescence intensity rapidly increased over time, reaching a plateau when the enzyme had converted all the substrate. In the second wash solution (blue), the recorded data did not show any significant change in the fluorescence intensity overnight. The "glass surface" data shows the fluorescence detected when a sample subjected to the two washes was placed in a FRET substrate solution, thus measuring the irreversibly immobilized enzyme activity (green). The control samples (magenta) include the FRET substrate in the absence of enzyme. b) Magnified view of the second wash and control data.

Taking advantage of having the enzymes immobilized on TEM grids, we employed electron microscopy to characterize the ability of the irreversibly immobilized enzyme to visualize the formation of any surface structures enabled by bio-catalytic self-assembly. 10 µl of Fmoc-T and F-NH_2 solution was pipetted on top of the grids and left overnight. Negative stain was applied and the samples were dried before TEM imaging. The images (Fig. 4) revealed the presence of a network of fibers on the grid surface, confirming that the immobilized enzymes were able to catalyze the conversion of the precursors into Fmoc-TF-NH_2, which then enabled their subsequent localized self-assembly into a thin mesh of nanofibrous structures.

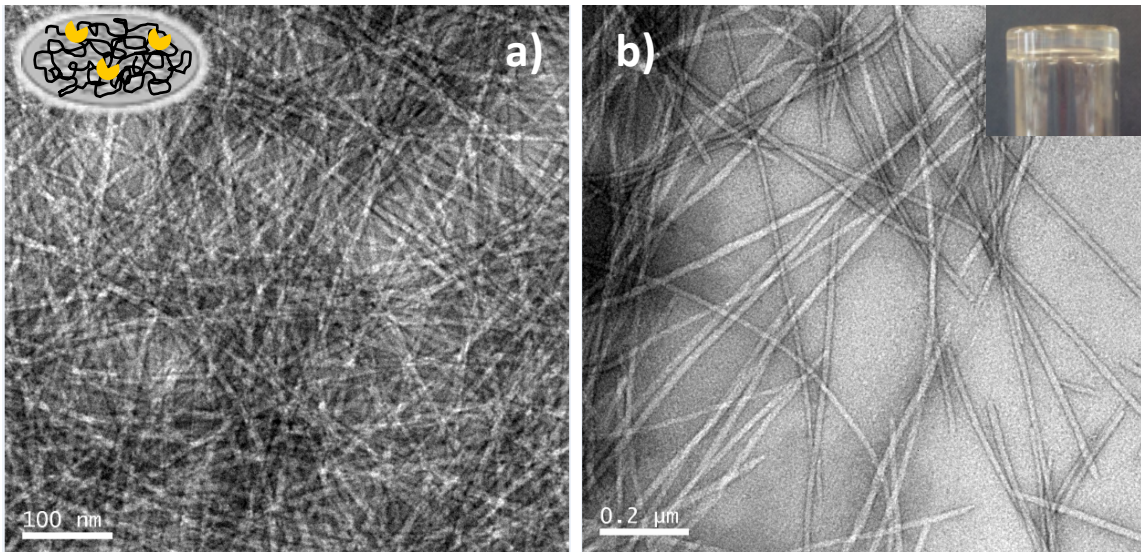

Figure 4 a) TEM images of fibers of Fmoc-TF-NH_2 grown on top of the carbon-coated copper TEM grids with immobilized thermolysin on polydopamine. b) Control: TEM image of fibers taken from a reference bulk gel formed from the same Fmoc-T and F-NH_2 pre-gelators using thermolysin dissolved in 1 mg/mL solution.

In this work, we employed novel polyphenol surface coatings for the immobilization of thermolysin, which are shown to be as effective as polydopamine coatings. We provide evidence that surface-mediated bulk gelation is not due to enzymes immobilized on the surface as previously suggested for a related system, [12] but is due to enzymes released into a pre-gelator solution. We propose that enzymes may be reversibly and irreversibly immobilized on a surface

and that the relative amounts of these populations may be controlled by the sample surface treatment. Although protein desorption (i.e. reversible immobilization) is a common phenomenon on the surfaces of a range of polymers, metals and metal oxides, there is no general rule on whether the conformation and activity of the released enzymes are preserved. On the other hand, common surface functionalization techniques using silanes, thiols, or bifunctional linkers are designed to efficiently attach proteins through irreversible covalent bonds and significant desorption/release of proteins is not expected. Reactions of polydopamine and polyphenols are relatively slow, [21] we were therefore able to exploit these coatings for (eventual) irreversible covalent binding as well as protein release. In our system, when the polyphenol/polydopamine coated samples undergo a mild washing procedure, reversibly bound thermolysin remaining on the surface can be released to catalyze the formation of the gelator Fmoc-TF-NH_2 in the solution covering the surface and enable formation of a bulk gel. When the samples undergo a more extensive washing procedure to ensure that loosely bound enzymes were removed, the remaining, irreversibly bound enzymes were still able to catalyze the conversion of the pre-gelators but only in close proximity to the surface. Our results indicate that our procedure led to the immobilization of half a monolayer of active enzymes, which enabled the localized formation of self-assembled nanofibers but no bulk gelation. This shows that enzyme (surface) localization provides a means to achieve spatial control of self-assembly as previously demonstrated for non-enzymatic catalysts,[30] and provides opportunities for the use of biocatalytic self-assembly for the formation of patterned nanostructures.

Supporting information: Preparation of the polymeric surface coatings and enzyme immobilization, FRET assay, additional TEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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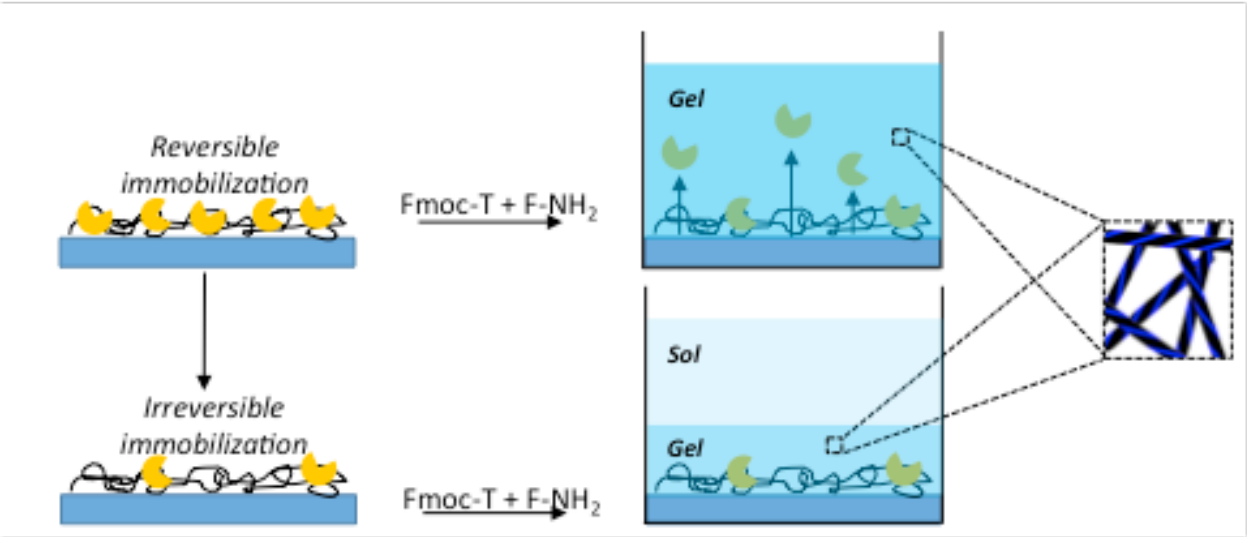
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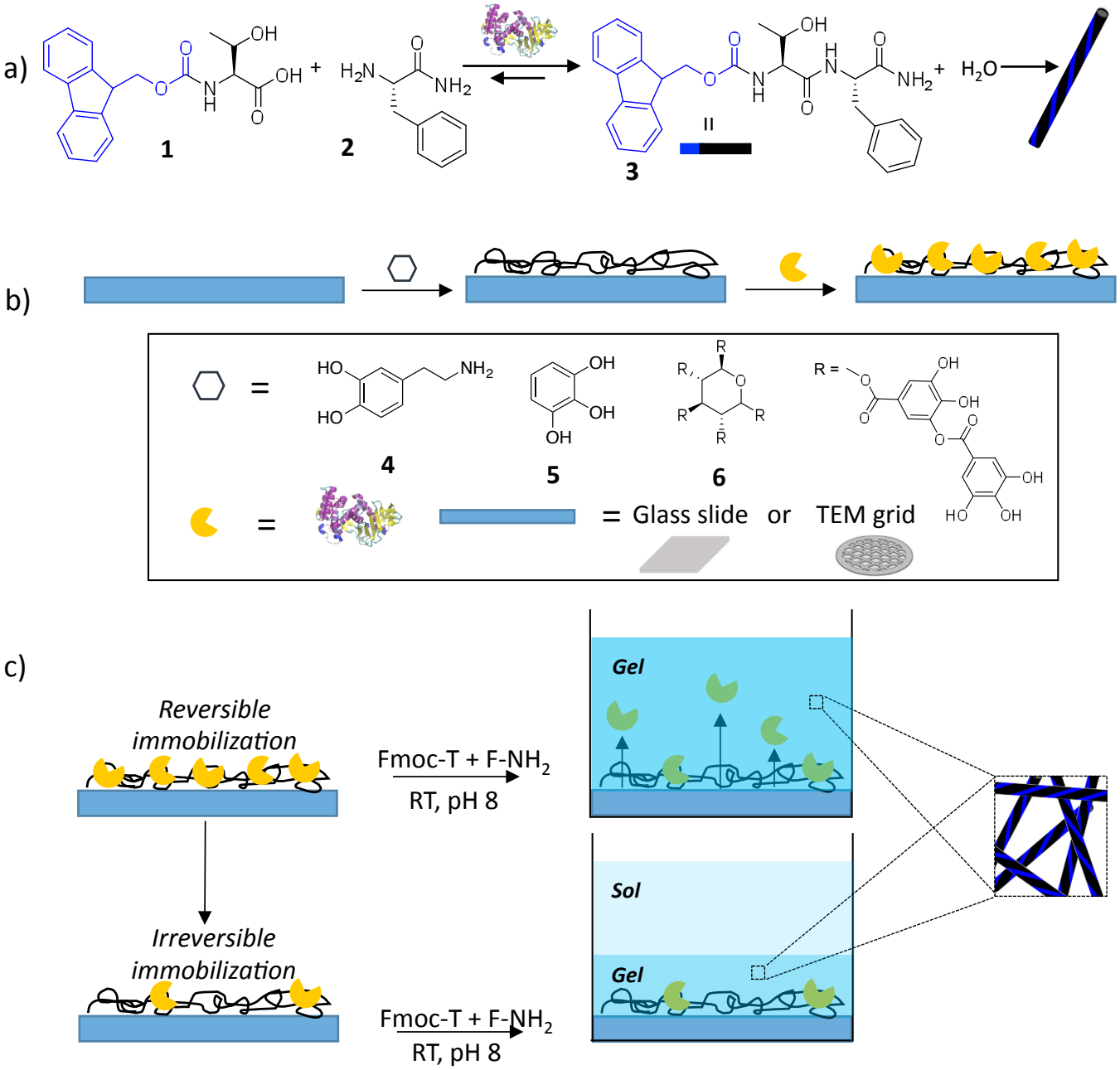
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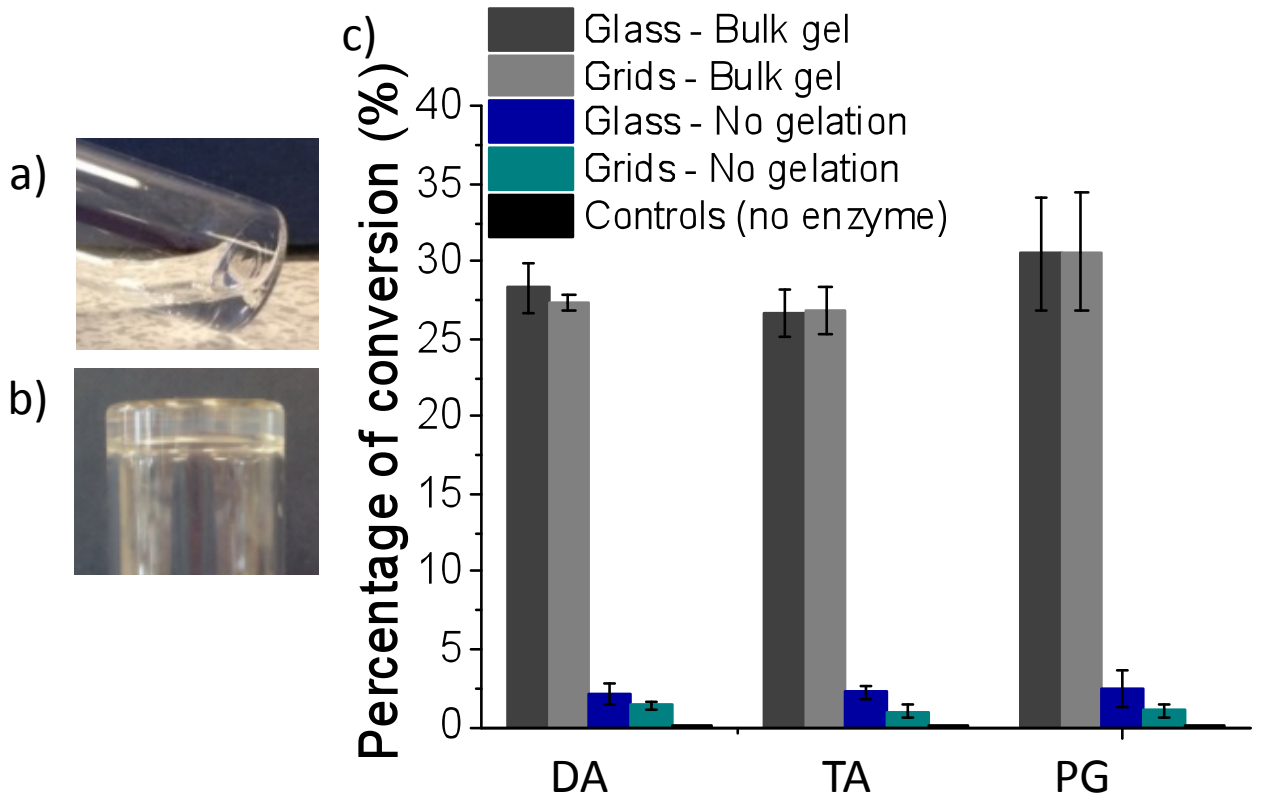
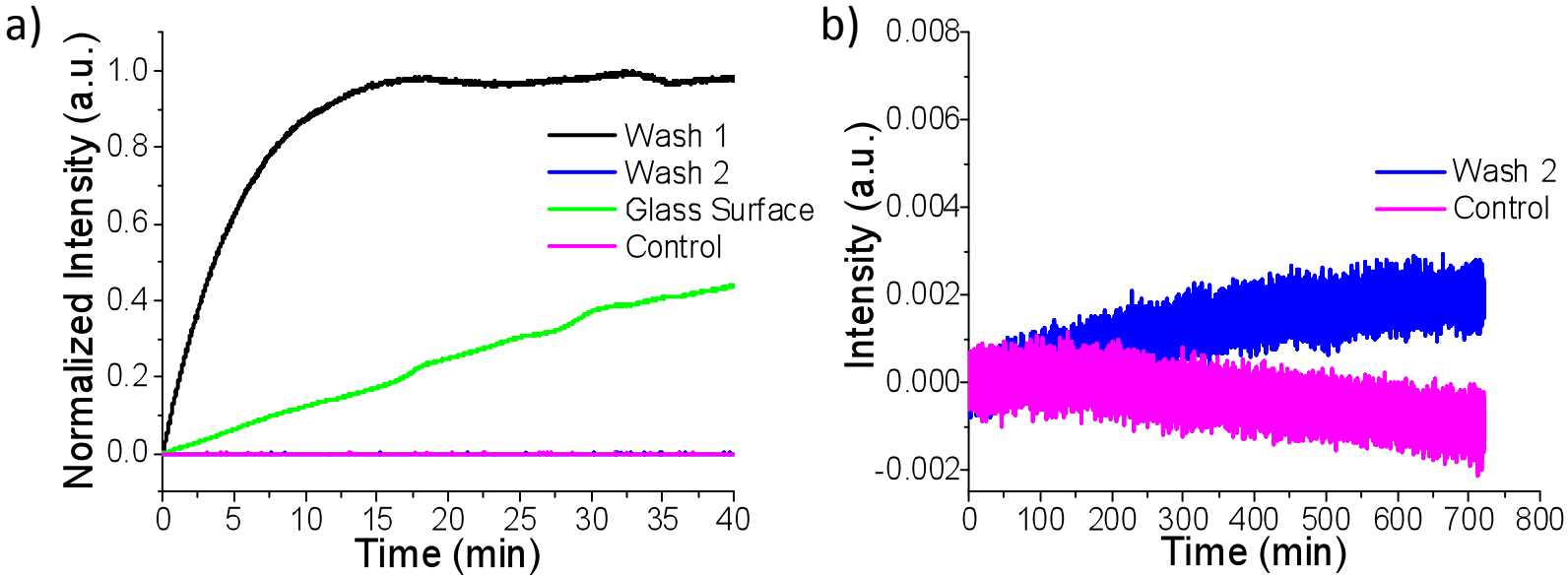
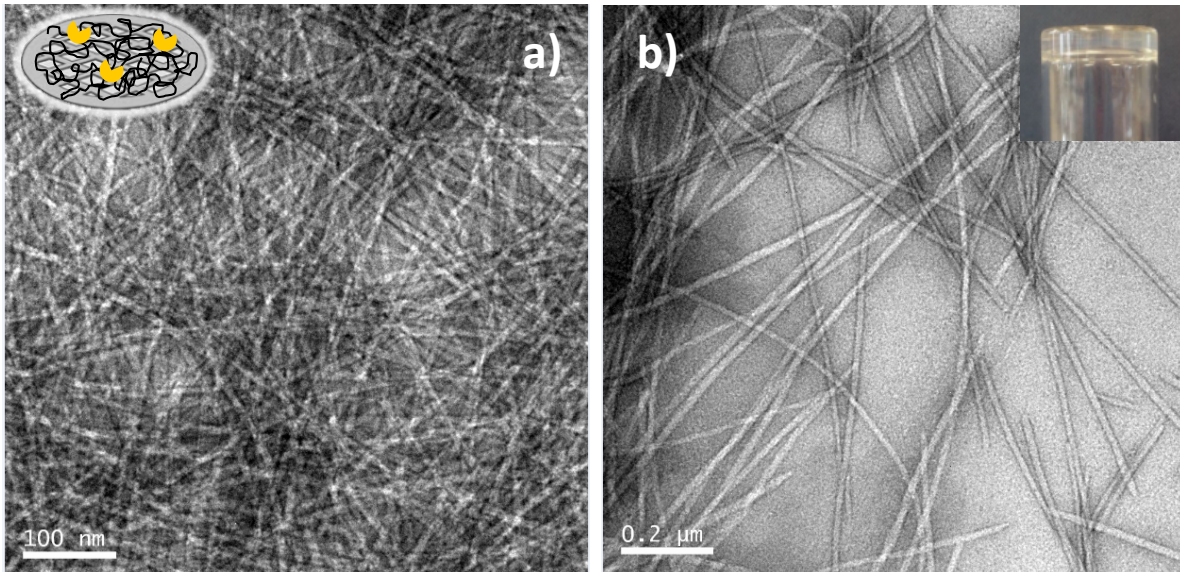
2
3
b)
4
5
6
= Glass slide or TEM grid
c)
Reversible immobilization
Irreversible immobilization
Fmoc-T + F-NH2
RT, pH 8
Fmoc-T + F-NH2
RT, pH 8
Gel
Sol
Gel


a)
b)
c)
Glass - Bulk gel
Grids - Bulk gel
Glass - No gelation
Grids - No gelation
Controls (no enzyme)
Percentage of conversion (%)
0
5
10
15
20
25
30
35
40
DA
TA
PG


a)
Normalized Intensity (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
Time (min)
Wash 1
Wash 2
Glass Surface
Control
b)
Intensity (a.u.)
0.008
0.006
0.004
0.002
0.000
-0.002
0
100
200
300
400
500
600
700
800
Time (min)
Wash 2
Control


a)
100 nm
b)
0.2 µm